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Exhibit I

#411

# Cholinesterases regulate neurite growth of chick nerve cells in vitro by means of a non-enzymatic mechanism

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**Abstract.** Cholinesterases present homologies with some cell adhesion molecules; however, it is unclear whether and how they perform adhesive functions. Here, we provide the first direct evidence showing that neurite growth in vitro from various neuronal tissues of the chick embryo can be modified by some, but not all, anticholinesterase agents. By quantifying the neuritic G4 antigen in tectal cell cultures, the effect of anticholinesterases on neurite growth is directly compared with their cholinesterase inhibitory action. BW 284C51 and ethopropazine, inhibiting acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), respectively, strongly decrease neurite growth in a dose-dependent manner. However, echothiophate which inhibits both cholinesterases, does not change neuritic growth. These quantitative data are supplemented by morphological observations in retinal explant cultures grown on striped laminin carpets, viz., defasciculation of neurite bundles by BW 284C51 and Bambuterol occurs, indicating that these drugs disturb adhesive mechanisms. These data strongly suggest that a) cholinesterases can participate in regulating axonal growth, b) both AChE and BChE can perform such a nonsynaptic function, and c) this function is not the result of the enzyme activity per se, since at least one drug was found that inhibits all cholinesterase activities but not neurite growth. Thus, a secondary site on cholinesterase molecules must be responsible for adhesive functions.

**Key words:** Acetylcholinesterase – Axon guidance – Butyrylcholinesterase – Cell adhesion molecules – HNK-1 epitope – Chicken

## Introduction

At the time when neuroblasts of the avian embryo cease to proliferate, the enzyme butyrylcholinesterase (BChE, EC 3.1.1.7) is transiently expressed, before acetylcholinesterase (AChE, EC 3.1.1.8) announces the postmitotic state (see review by Layer 1990; Willbold and Layer 1992). Only thereafter do neurones extend long axons (Weikert et al. 1990). Although far from being restricted to nervous tissues (Drews 1975), AChE is only known to split the neurotransmitter ACh at cholinergic synapses. BChE is coded for by a separate gene (Prody et al. 1987; Chatonnet and Lockridge 1989); a defined function for BChE is not yet known (Silver 1974; Massoulié and Bon 1982). There is suggestive evidence that BChE is implicated in the regulation of the proliferative state of certain embryonic (for a review, see Layer 1991a) and tumor cells (Lapidot-Lifson et al. 1989). Moreover, BChE may play a role in regulating the expression of AChE (Koelle et al. 1976; Layer et al. 1992). Particularly intriguing is the occurrence of BChE in areas that shortly thereafter will be invaded by axons during generation (Layer et al. 1988; Layer and Kaulich 1991) or regeneration (Haninec and Dubovy 1992) of peripheral nerves, suggesting that BChE is involved in neurite guidance.

Neurite guidance can be conveyed by a number of cell adhesion molecules (CAMs), including the IgG-like CAMs and the cadherins (Takeichi 1988). In particular, fasciculation molecules such as L1, G4 and TAG-1 (for a review, see Rathjen 1991) are crucial for the proper outgrowth of neurites and for keeping them together within organized fiber bundles. However, our knowledge about CAMs is incomplete, as more CAMs are still anticipated. A widespread feature of CAMs is the HNK-1 sugar epitope (Kruse et al. 1984) that may be functionally significant (Cole and Schachner 1987). Noticeably, AChE from *Torpedo* (Bon et al. 1987) and BChE from chicken serum and brain (Treskatis et al. 1992, unpublished data) bear the HNK-1 epitope. Moreover, neurotactin and glutactin are CAMs from *Drosophila* and share sequence homologies with cholinesterases (De la Escalera et al. 1990; Barthalay et al. 1990; Krejci et al. 1991). All these data lead to the question whether cholinesterases can function as CAMs, e.g. by being involved

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in neurite growth regulation. Can both AChE and BChE perform such a function? If so, is the active center directly involved, or are there secondary noncholinergic sites on these molecules?

In this study, we have approached these questions by taking advantage of the highly specific action of a series of anticholinesterase agents and have tested whether they can modify neurite outgrowth under in vitro conditions. Some of these agents are of therapeutical use, others have become widely known as nerve gases, insecticides, and pesticides (Silver 1974). All of those that have been used in this study are considered exclusively to affect cholinesterases, and thus, if effects are seen, they must be caused by cholinesterases. For detecting quantitative changes of neurite growth, we have cultured tectal cells on microplates; the extent of neurite growth has been measured by an enzyme-linked immunosorbent assay (manuscript in preparation) for the neurite-specific G4 antigen (Rathjen et al. 1987). To evaluate morphological changes of neurites, we have applied the stripe assay (Walter et al. 1987). The results show that both types of cholinesterases can play a neurogenetic nonsynaptic role in regulating neurite growth. Some of these results have been presented in preliminary form (Layer 1991 b).

## Materials and methods

### *Cholinesterase inhibitors*

Tetraisopropylpyrophosphoramide (iso-OMPA; Sigma, Deisenhofen, Germany), a specific inhibitor of BChE, was prepared as a 10 mM solution in Hanks' medium. BW 284C51, 1,5-bis-(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (Sigma), a specific inhibitor of AChE, was prepared as a 50 mM solution in Hanks' medium. Bambuterol hydrochloride (Tunek and Svensson 1988), a highly selective, reversible, noncompetitive inhibitor of human serum BChE (item No 22-165-13, AB Draco, gift of Dr. L.A. Svensson, Lund, Sweden) was prepared as 0.1 M solution in water. Echothiophate, an irreversible, non-selective cholinesterase inhibitor, was prepared as 10 mM solution in Hanks. All stock solutions were stored at 4° C.

### *Tectal cell cultures on poly-L-lysine-coated microdishes*

Tecta from E5 chicken embryos (White Leghorn) were isolated, washed in Hanks' medium, and collected in F12-medium on ice. For dissociation, the tecta were centrifuged for 5 min at 1000 rpm and then treated with 100 µg/ml trypsin (Worthington, Freehold, No. LS 030707) for 4 min at 25° C. The tissues were centrifuged for 5 min at 1000 rpm, washed once in Hanks, again centrifuged (as above) and then gently dissociated into single cells (10–20 strokes) with a fire-polished Pasteur pipette in the presence of 2 ml F12 medium plus 60 µl DNase (50 µg/ml 0.15 M NaCl, Worthington No. LS 06330). Centrifugation for 10 min at 1000 rpm, a wash in Hanks plus 10 µl DNase, and two further washes without DNase followed. Cells were then suspended in N2-medium (Bottenstein and Sato 1979) and plated on poly-L-lysine-coated microwells (Costar, Cambridge, Mass., Cluster 24 wells, 3424 Mark II; pretreatment of wells: 500 µl of a 100 µg/ml distilled water solution of poly-L-lysine, type V, Sigma P-7890, overnight at 37° C; then two times washed with Hanks' medium). Cell density was adjusted to $1.4–1.6 \times 10^6$ cells/well. The final volume/well was 0.8 ml. All experiments were run in triplicate or quadruplicate; all series were repeated several-fold with similar results. The dishes were incubated in Heraeus incubators (37° C, 95% air/5% $CO_2$).

### *Determination of AChE activities in cell homogenates*

At the indicated stages, the tectal cells from individual wells were washed several times in phosphate-buffered saline (PBS), and then 200 µl homogenization buffer (1 mM $NaHCO_3$, 0.2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM spermidine, pH 8.0) were added; the cells were scratched off from the wells and collected. The samples were homogenized by a 15-s sonification step on ice (setting 4, Sonifier B-12, Branson Sonic Power Company, Danbury, Conn.). If not otherwise stated, crude homogenates from individual culture dishes were used for enzyme, protein, and G4 antigen determinations. Enzyme activities were measured according to the Ellman procedure at 412 nm (Ellman et al. 1961). The substrate concentration for acetylthiocholine iodide was 1.5 mM. AChE was determined in the presence of 0.1 mM iso-OMPA to inhibit any BChE activity. The protein content was measured with the Lowry method, using bovine serum albumin as a standard (Lowry et al. 1951).

### *Quantitative determination of neurite growth by an ELISA assay for the G4 antigen in tissue homogenates*

The test as established earlier (manuscript in preparation), represents a double-sandwich system, using an excess amount of monoclonal antibodies (mAb) against G4 as bottom substrate, ensuring fixation of the antigen to the plate. A highly purified polyclonal G4 rabbit antiserum is used as the primary detection antibody, which in turn is detected with an HRP-coupled anti-rabbit antibody. The assay is highly sensitive, allowing for the detection of minute amounts of G4 antigen in the picogram range. At the same time, this assay is highly dependent on the individual procedures. Therefore, it is absolutely essential to run individual calibration curves with each ELISA plate.

Each assay was performed in a new microtiter plate (Nunc, 96 wells, flat bottom). Each well was incubated with 190 µl anti-G4-mAb overnight at 4° C (gift of Dr. F. Rathjen, Hamburg; stock of 6 mg/ml; vial 12-J-4E-3-1, MSPBS 1.4.87; 10 µl monoclonal antibody against G4 diluted in 20 ml $Na_2CO_3$ buffer). Then, each well was washed for 10 min in PBS/Triton (PBS including 0.5% Triton X-100) at room temperature (RT), 1 h in PBS/Triton/BSA (PBS including 0.5% Triton X-100 and 1% BSA, inactivated for 30 min at 56° C) at 37° C and then twice for 10 min in PBS/Triton at RT (225 µl each; = washing protocol 1). Then followed the binding of the G4 antigen. Either 50 µl sample or appropriately diluted sample plus 150 µl PBS/Triton were pipetted into each well and incubated for 2 h at RT. In parallel, a calibration curve was determined for each individual assay (we used dilutions of a highly purified 29 µg/ml G4 stock solution, gift of Dr. F. Rathjen, Hamburg in PBS/Triton). After antigen incubation, the supernatants were removed. Each well was washed for 10 min in PBS/Triton, for 20 min in PBS/Triton/BSA, and three times for 10 min in PBS/Triton (each 225 µl/well; = washing protocol 2). To detect the G4 antigen, each well was incubated with 190 µl Ab2 (20 µl Fab-rabbit-anti-G4 in 20 ml PBS/Triton; vial 4003, 5 mg/ml) overnight at 4° C. A series of washes (protocol 1) was performed, before incubation the combination of 10 µl HRP-coupled mouse-anti-rabbit IgG (Jackson Immuno Research, West Grove, Pa., Code 211-035-109) plus 20 µl of 2.9 mg F11 mAb dissolved in 20 ml PBS/Triton (gift Dr. Rathjen, Hamburg, stock 1-5-711-3-2-5, SpA-El Asc 24.6.86) incubated for 2 h at RT. The F11 antibody was added here to suppress nonspecific binding of HRP-coupled second antibody to the mAb/G4 on the bottom of the well. Four washes in PBS/Triton for 15 min each and 1 wash in citrate buffer for 10 min were now necessary. For the HRP reaction, 8 mg o-phenylene-diamine were

dissolved in 20 ml 0.2 M citrate buffer, pH 5.5 (Sørensen). To 13 ml of this solution, 3 µl 30% $H_2O_2$ were added; 190 µl were then added per well and reacted for 5 min in the dark at RT. The reaction was stopped by adding 50 µl 6N $H_2SO_4$. The color extinction was determined in an ELISA reader at 430/570 nm.

### *Retinal explant cultures on laminin stripes*

The culture of retinal explants on perpendicularly oriented striped laminin carpets was performed as described (Walter et al. 1987). Briefly, an isolated E6 chicken eye was transferred to a ConA-pretreated cellulose nitrate filter (Sartorius, Göttingen; No. 13006-50-ACN). The lens and the vitreous body were removed. The retina was spread on the filter, with the ganglion cell layer facing upwards. Retinal stripes of 0,2 mm width were cut with a tissue chopper (McIlwain) and then transferred, with the tissue oriented downwards, onto laminin-coated coverslips (for preparation, see below). The edges of the retinal stripes were fixed by metal weights. A volume of 2 ml F12-based medium (10% fetal calf serum, 2% chicken serum, 2 mM glutamine, 10 units/ml penicillin/streptomycin plus 0.4% methyl-cellulose) was added, and dishes were incubated at 37° C and 4% $CO_2$.

For the preparation of laminin stripes on glass coverslips, a silicon filtering device provided a set of 40 to 50 µm-thick parallel horizontal channels (gift of Dr. F. Bonhoeffer, Tübingen). A coverslip was placed on top. The device is injected with a laminin solution (100 µg/ml PBS, laminin from EY Labs, San Mateo, Ca., No. 2404, Medac) and incubated for 1 h at 37° C in a moist chamber. After washing, the coverslip was gently separated from the device. The glass coverslip was now covered with a series of 40 to 50 µm thick parallel laminin stripes.

## Results

### *Quantitative changes following treatment with anticholinesterase drugs*

Dissociated tectal cells from 5-day-old chick embryos were plated on poly-L-lysine-coated microplates (24 wells/dish), allowing large numbers of parallel experiments. Tectal cells were cultured in a serum-free medium; thus, all cholinesterase activity present in the system was produced by the cells. After 2 days, neurite growth was pronounced showing a network of fibers, often connecting islands of aggregated cells (Fig. 1, Control). In order to quantify the action of anticholinesterases on neurite outgrowth, the cellular material including cells and neurites could easily be scratched off from a single well. From the homogenate, it was then possible to determine the total protein and the AChE activity.

Using the same homogenates, we have applied an ELISA assay to detect the neurite-specific G4 antigen that belongs to the family of IgG-like adhesion molecules (Rathjen et al. 1987). This antigen is predominantly distributed on a large number of early forming neuritic systems (Weikert et al. 1990), including axons extending from retinal ganglion cells and tectal cells. Since the G4 antigen is regularly distributed along the neurite shafts, determination of the G4 antigen gives a good measure of the amount of neuritic material present in the tissue (we are not claiming this to be precisely proportional to the actual neurite lengths, because of the changing geometry of neurites during growth, see Dodd et al. 1988). Our assay allows for the detection of minute amounts of G4-antigen in the picogram range.

We have applied a series of anticholinesterases that are known to bind in a distinct manner selectively to either one or both of the cholinesterases (see 'Materials and methods' for a list of the anticholinesterases). When applying these agents to the microplates, pronounced effects on the production of the G4 antigen become evident with some of them. The effect of a wide range of drug concentrations on neurite growth and AChE activity are shown in Fig. 2 (note the high drug concentrations are on the left). Ethopropazine, which is known


Fig. 1. Neurite growth of 5-day-old chicken tectal cells is changed by some but not all anticholinesterase drugs. Whereas *ethopropazine* (10 µM) is a potent inhibitor of neurite growth, *echothiophate* (50 µM) is not. Tectal neurites express the G4 antigen. The amount of G4 antigen as a measure of neurite growth and the AChE activity expressed in tectal cells cultured on microplates can be determined simultaneously from homogenates (see Fig. 2). *Bar:* 50 µm

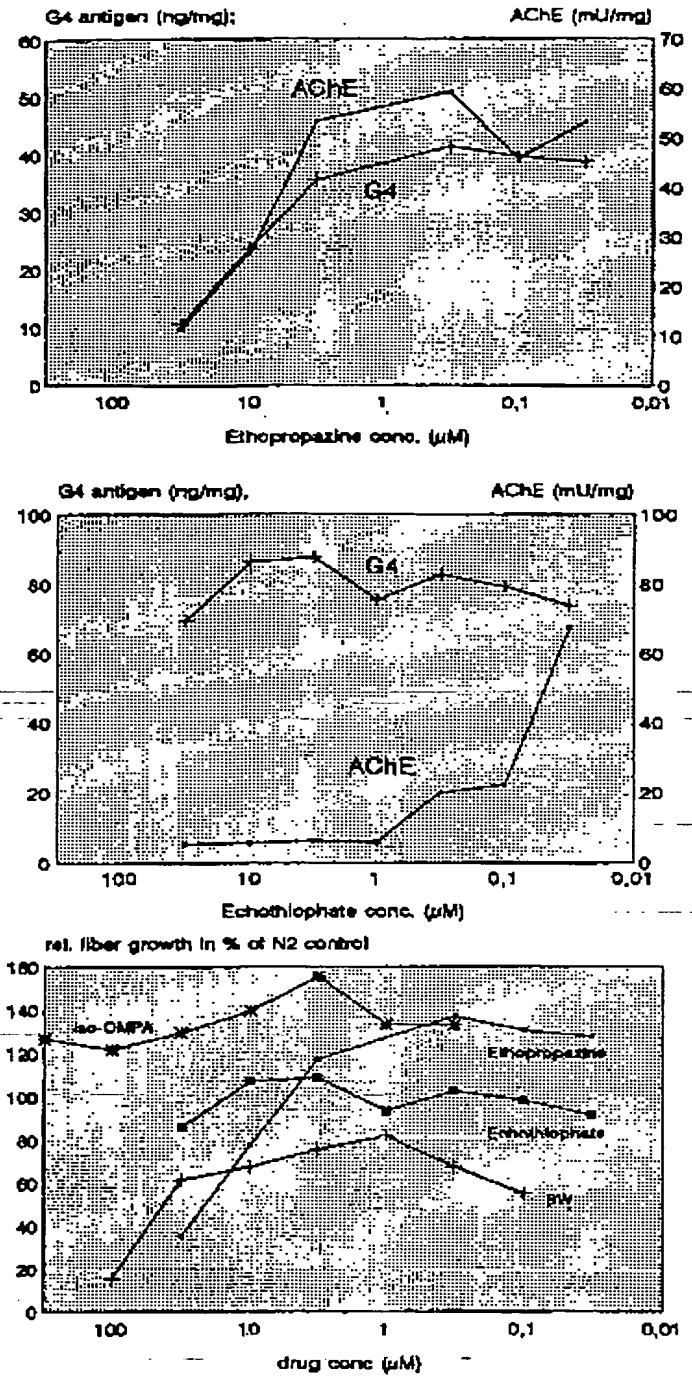


Fig. 2. Quantitative changes of neurite growth by some, but not all anticholinesterase agents, demonstrating the dependence of the effects on the cholinesterase molecules, but not strictly on their enzyme activity. *Top* Concentration-dependent inhibition of neurite growth by the BChE inhibitor ethopropazine, as determined in homogenates of tectal cells cultured in N2-medium for 2 days in microplates (drug concentration decreases from *left* to *right*). Neurite growth is measured by an ELISA assay detecting the neurite-specific antigen G4 (Rathjen et al. 1987). AChE activities are determined from the same tissue samples. Note that above 1 μM, both G4 and AChE are affected similarly. *Middle* In contrast, echothiophate blocks all cholinesterase activity (only AChE is shown here), but does not inhibit neurite growth. *Bottom* G4 expression in tectal cell cultures in the presence of four different cholinesterase inhibitors is plotted as percent fiber growth compared with untreated N2-controls. Note that *echothiophate* and the BChE inhibitor *iso-OMPA* have no or only slight effects, whereas BW 284C51 (BW) and *ethopropazine* decrease neurite growth. Multiple series of experiments gave similar results; means of triplicate dishes of one series are plotted

to be a selective competitive inhibitor of BChE (Silver 1974; our own observations), inhibits neurite growth in a concentration-dependent manner at concentrations above 1 μM (Fig. 2, top; see also Fig. 5). At the same time, this drug leads to a full inhibition of BChE (data not shown), and to a decrease in cellular AChE (Fig. 2, top) (for the regulation of AChE by BChE activity, see Layer et al. 1992). Another example of a cholinesterase inhibitor that inhibits neurite growth is the water-soluble reversible AChE inhibitor BW 284C51 (Fig. 2, bottom). At 0.1 mM, neurite growth is inhibited by less than 20%.

Noticeably, not all cholinesterase inhibitors affect neurite growth. Echothiophate, which is a potent blocker for both AChE and BChE activities, does not affect neurite growth from 50 nM to 50 μM (Fig. 2, middle; AChE is fully inhibited above 1 μM; data for BChE are not shown). Iso-OMPA, which is a lipophilic, nearly irreversible, competitive BChE inhibitor, represents a second example of an anticholinesterase agent that does not inhibit neurite growth (Fig. 2, bottom; the slight increase of neurite growth by iso-OMPA may be nonspecific, since it is not dose-dependent).

The actions of all four drugs on neurite growth are plotted as percent fiber growth in Fig. 2 (bottom). Thus, we have found two cholinesterase inhibitors that inhibit neurite growth (ethopropazine and BW 284C51), and two inhibitors (echothiophate and iso-OMPA) that do not affect neurite growth. In particular, echothiophate inhibits all cholinesterase activities (including cell-internal) in our systems, but has no severe impact on neurite outgrowth.

### *Morphology of retinal neurite outgrowth: anticholinesterases affecting fasciculation of neurites*

In order to evaluate the fasciculation of neurite bundles, we have studied the outgrowth of retinal fibers from retinal explants on striped laminin carpets. The stripe assay offers a number of advantages over conventional cultures: since all fibers tend to stay on the applied laminin substrate stripes, the outgrowing neurites form nearly parallel bundles (Fig. 3). In such a system, it is easy to measure growth rates of neurites, and to detect whether they stick together or defasciculate. In control experiments, neurites extending from the retinal explant form bundles (fascicles composed of hundreds of individual neurites) at a distance of 40–50 μm from each other (Figs. 3, 4, Control). Since outgrowing neurites from nasal and temporal areas show markedly different

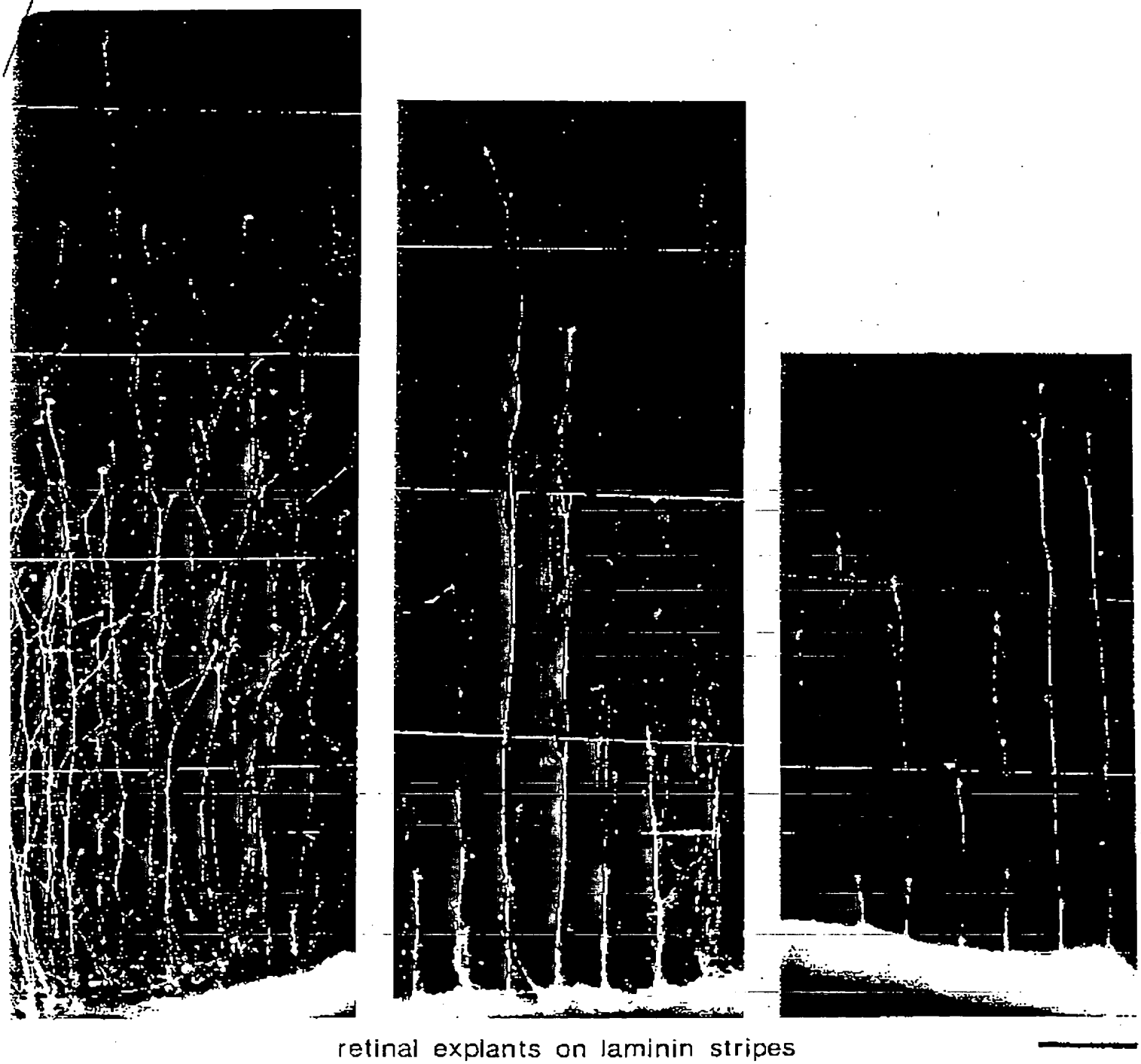


Fig. 3. Anticholinesterases change the morphology of retinal explant neurites when grown on the laminin stripe assay (Walter et al. 1987). Stripes of E6 retinae cut in a naso-temporal orientation (explants are visible at the *lower* edge of the micrographs) are explanted on coverslips that present 40 to 60 μm wide parallel laminin stripes in a perpendicular direction. Specific inhibitors of AChE (BW 284C51, 50 μM, *right*) and of BChE (iso-OMPA at 100 μM, *left*) are added; cultures are shown after 1 day. Neurites are visualized by binding of the G4 antibody plus rhodamine-isothiocyanate-coupled second antibody. Note that BW 284C51 leads to shorter and thinner neurite bundles; iso-OMPA slightly increases neurite growth with longer and less organized bundles (cf. also Fig. 2). *Bar*: 150 μm

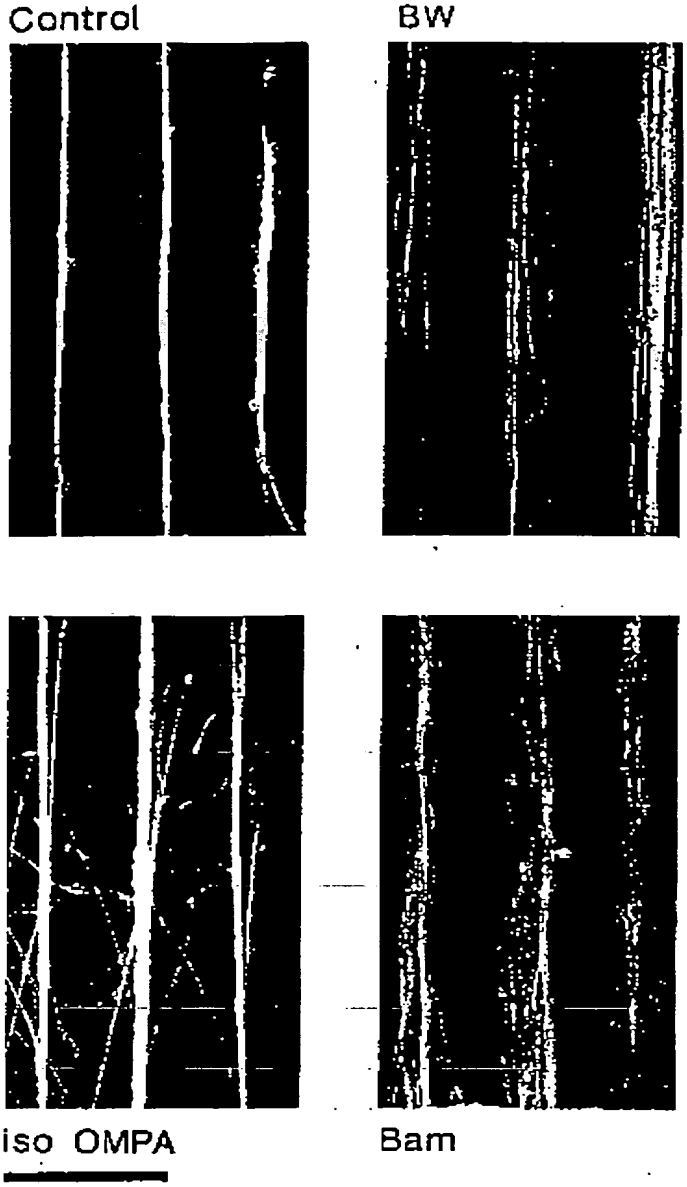


**Fig. 4.** Defasciculation of neurite bundles during anticholinesterase treatment, as revealed in micrographs of retinal neurites in the laminin stripe assay after 1 day culture. In addition to decreasing neurite growth (cf. Fig. 3), BW 284C51 induces defasciculation of bundles (*upper right*). Similar defasciculation is induced by the BChE inhibitor bambuterol (*Bam*; *lower right*). In the presence of iso-OMPA, neurite bundles tend to be thicker and less organized (*lower left*). *Bar:* 150 μm

morphologies (Halfter et al. 1983), we have consistently compared neurite outgrowth from temporal regions.

Anticholinesterase drugs added to the culture media distinctively change the morphologies of the outgrowing neurite bundles, depending on the specific drug applied. Upon application of the AChE inhibitor BW 284C51, the neurite bundles tend to be shorter and somewhat thinner (Fig. 3, right). Often, particular stretches of the fiber bundles defasciculate in the presence of BW 284C51 (Fig. 4, upper right). Bambuterol is a reversible, remarkably selective, potent inhibitor of human BChE (Tunek and Svensson 1988); it also inhibits chicken BChE (unpublished). Its action on neurite outgrowth is similar to that of the AChE inhibitor BW 284C51, with defasciculation being even more pronounced (Fig. 4, lower right). In addition, the growth cone morphology is severely changed (not shown). Neurite growth in the presence of iso-OMPA is effluent (Fig. 3, left; Fig. 4, lower left). Fiber bundles are longer than in the control (cf. Fig. 2, bottom). Typically, disorientation of neurites on the laminin stripes and bridging between neighboring stripes is abundant. Corresponding changes have been detected in other cellular systems, particularly with explants of neural tube on laminin carpets, and in explant cultures of dorsal root ganglia on poly-L-lysine-coated dishes (data not shown). This indicates that the proposed function of cholinesterases on neurite outgrowth is not restricted to retina and tectum, but rather is general.

## Discussion

The inhibition of neurite growth by BW 284C51, an inhibitor of AChE, and by ethopropazine, an inhibitor of BChE, strongly indicates that both types of cholinesterases can regulate neurite growth. Our extensive studies on the regulation of expression patterns of both cholinesterases during the development of the avian nervous system have shown that these enzymes are present on a wide number of early neurones and their neurites (see Introduction); this has prompted us to search for neurogenetic functions of cholinesterases. Our present results are a first important step to showing that the effect of cholinesterases on growth does not depend directly on their enzymatic activities, but may reside in a different epitope of the cholinesterase molecule (see scheme in Fig. 5). This notion is strongly supported by our finding that the cholinesterase inhibition by echothiophate and iso-OMPA does not inhibit neurite growth (iso-OMPA even slightly stimulates growth). It is well known that different cholinergic ligands and anticholinesterase drugs interact differently with the active site and the various peripheral sites on cholinesterase molecules (Layer et al. 1976; for a review, see Hucho et al. 1991). Thus, it is likely that those drugs that show effects on neurite growth exert their action by primarily interacting with such peripheral sites that in turn must then be involved in the adhesive function of cholinesterases. Recent observations that cholinergic ligands show effects on growing neurites from retinal ganglion cells in vitro (Lipton et al. 1988) can be explained accordingly.

Thus, provided that the neurite-regulating pool of cholinesterases is indeed reached and blocked by the drugs in our experiments, then the echothiophate result must mean that the activity of the choline-splitting enzyme does not per se induce the neurite changes. If so, the predominant neurite-related action must be taken by another part of the cholinesterase molecule, such as the HNK-1 epitope or sequences that are homologous

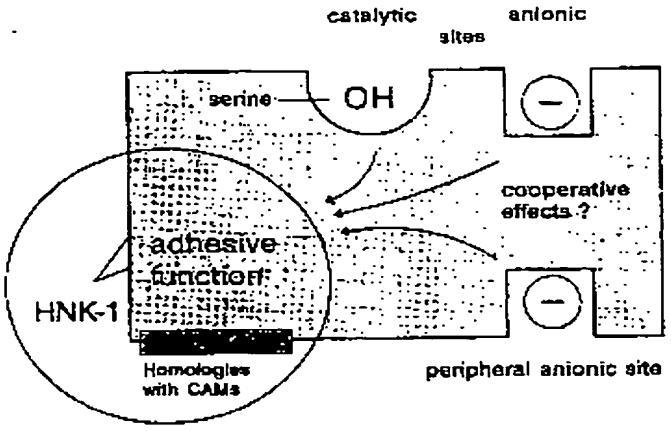


Fig. 5. A scheme for the proposed multifunctionality of a cholinesterase protein subunit. The *encircled* region includes sequence homologies with CAMs plus the HNK-1 sugar epitope; this part may be responsible for adhesive functioning. Depending on the binding of particular anticholinesterase agents to the central or peripheral binding sites (Layer et al. 1976; for a review, see Hucho et al. 1991), steric interaction with this secondary function of cholinesterases may or may not occur. Accordingly, echothiophate does not interact, whereas BW 284C51 and ethopropazine do interact, with this secondary site

with CAMs (see Introduction and scheme in Fig. 5). Recent X-ray crystallographic results have refined our views of the structure of cholinesterases (Sussman et al. 1991); such studies may also help to elucidate whether occupation of the various binding sites can cooperatively influence the adhesive function (see scheme in Fig. 5).

By using the sensitive stripe assay, we have shown here that some anticholinesterase agents can exert defasciculating effects on neurite bundles. Since we have observed distinct morphological changes for each individual anticholinesterase agent, it is unlikely that the effects can be attributed to nonspecific side effects possibly caused by interaction of the agents with other serine hydrolases. Moreover, we have no indications that cell viability was affected under the culture conditions as applied in this study. This strongly indicates that cholinesterases are specifically involved in cell adhesion phenomena.

Our measurements on tectal cell cultures have allowed us to correlate quantitatively the inhibitory action of anticholinesterase agents on cholinesterase activity with that on neurite growth. Since not all cholinesterase-blocking drugs have been found to inhibit neurite growth, it is highly likely that a secondary site on cholinesterases is involved in regulating neurite growth. Further work has now to define the molecular structure of the adhesive domains of cholinesterases.

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